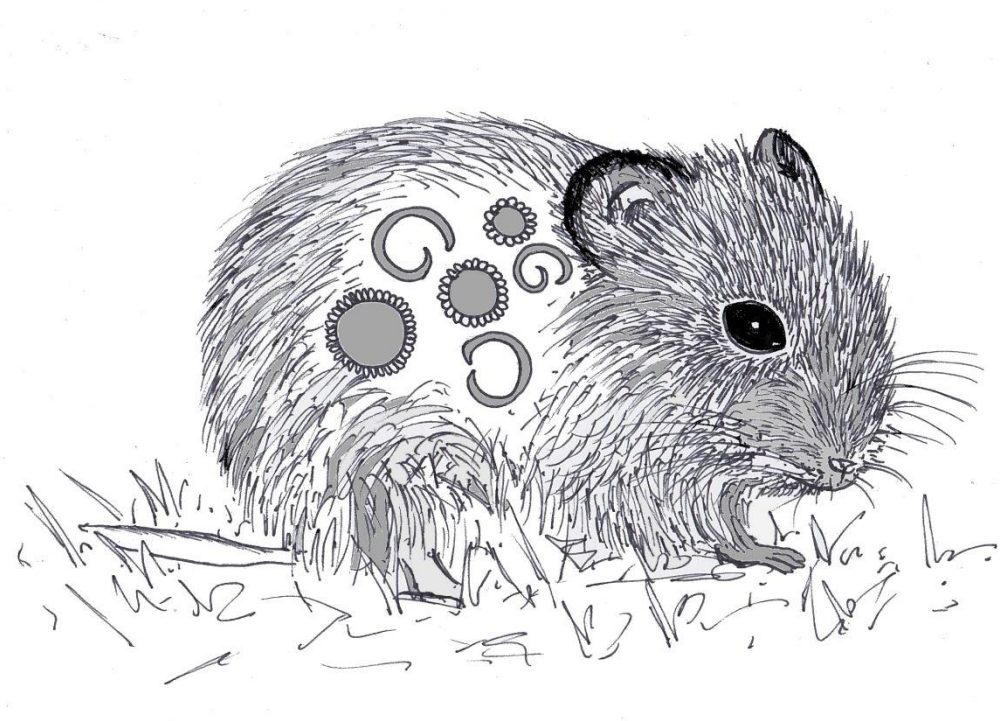
Puumala Hantavirus and Helminth Coinfections in Wild Bank Voles

Paula Ahola



Licentiate thesis

Faculty of Veterinary Medicine

University of Helsinki

2020

HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Tiedekunta - Fakultet - Faculty	Osasto - Avdelning – Department	
Eläinlääketieteellinen tiedekunta	Eläinlääketieteellisten biotieteiden osasto	
Tekijä - Författare - Author		
Paula Ahola		
Työn nimi - Arbetets titel - Title		
Puumala Hantavirus and Helminth Coinfections in Wild Bank Voles		
Oppiaine - Läroämne - Subject		
Virologia		
Työn laji - Arbetets art - Level	Aika - Datum - Month and year	Sivumäärä - Sidoantal - Number of pages
Lisensiaatin tutkielma	2/2020	34

Tiivistelmä - Referat – Abstract

Puumala hantavirus (PUUV) is the zoonotic pathogen of nephropathia epidemica (NE) known as myyräkuume in Finnish. PUUV hasn't been reported to spread from human-to-human but the risk for human infection is directly correlating with the prevalence of PUUV in its natural host bank vole. Until now research has mainly focused on a one-host-one-pathogen framework, even though in natural systems individuals are usually coinfected with multiple species of parasites. An antagonist relationship between the immune pathways T-helper-1 (Th-1) and T-helper-2 (Th-2) is well studied in laboratory experiments. Th-1 directs immune responses against intracellular organisms such as viruses and Th-2 extracellular organisms such as helminths. These two pathways can't fully function at the same time, leading usually to Th-1 or Th-2 biased immune response depending on the pathogen. A large field survey was conducted to analyze how concurrent helminth infections influence the prevalence and viral loads of PUUV in individual bank voles, and thereby influence human exposure to this virus. My hypothesis was that helminths induce a Th-2 biased immune response, leading to higher PUUV prevalence and viral loads in helminth infected voles than in uninfected voles. In contrary to my original hypothesis, intestinal helminths seemed to have a protective effect on voles acquiring PUUV infection and the disease burden. These findings were consistently shown through serology and PCR results from kidney and lungs. An explanation to this finding could be that helminths cause tissue damage in the intestinal wall, thus predisposing voles to secondary intracellular infections and enhanced Th-1 immune response. This enhanced Th-1 immune response could then protect the vole from new intracellular infections such as PUUV-infection. Coinfections are occurring everywhere in the wildlife systems and shouldn't be neglected in the future disease management, especially when talking about zoonoses that pose a risk to humans. There can be surprising outcomes when multiple parasites share the same host, such as was found in this study, and these can lead to unintentional consequences of disease control procedures. More research and larger sample pools are needed to reinforce these results and deepen our knowledge in the immunology of coinfections.

Puumala hantavirus (PUUV) on zoonoottinen virus, joka leviää metsämyyrien ulosteen saastuttaman pölyn välityksellä ja aiheuttaa ihmisessä myyräkuumetta. Koska se ei leviä ihmisestä ihmiseen, on tärkeää ymmärtää sen leviämisen dynamiikkaa myyräpopulaatioissa, jotta voidaan kontrolloida myös ihmisten altistumista virukselle. Luonnossa metsämyyrät altistuvat valtavalle määrälle erilaisia taudinaiheuttajia, ja yhteistartunnat useamman eri taudinaiheuttajan kanssa samanaikaisesti ovat hyvin yleisiä. Eri taudinaiheuttajien yhteistartunnat ovat jääneet etenkin villieläimissä vähälle huomiolle, vaikka ne voivat vaikuttaa ratkaisevasti ymmärrykseemme zoonoottisten tartuntojen leviämisestä. Laboratoriokokeissa paljon tutkittu antagonistinen suhde auttaja-T-solujen (Th-solu) indusoimien Th-1 ja Th-2 -soluvasteiden välillä vaikuttaa yhteistartuntojen immunologian taustalla. Th-1 -soluvaste ohjaa immuunireaktioita intrasellulaarisia taudinaiheuttajia, kuten viruksia vastaan, kun taas Th-2-soluvaste ekstrasellulaarisia taudinaiheuttajia, kuten suolistoloisia vastaan. Nämä kaksi soluvastetta tasapainottelevat keskenään niin, että toisen soluvasteen voimistuessa toinen heikkenee. Analysoin noin 130 metsämyyrän suolistoloisten sekä PUUV:n välisiä yhteistartuntoja selvittääkseni, miten samanaikaiset suolistoloistartunnat vaikuttavat PUUV:n esiintyvyyteen ja tartunnan voimakkuuteen yksittäisissä metsämyyrissä. Hypoteesini oli, että suolistoloistartunta voimistaa Th-2-soluvastetta heikentäen samalla Th-1-soluvastetta, ja täten lisää myyrän herkkyyttä PUUV-tartunnalle sekä lisää tartunnan voimakkuutta. Hypoteesin vastaisesti suolistoloistartunnalla tuntui olevan suojaava vaikutus myyrien PUUV-tartuntoja vastaan. Tämä ilmeni johdonmukaisesti sekä serologian että elinnäytteiden PCR-tulosten kautta. Mahdollinen selitys löydökselle on suolistoloisten aiheuttama kudosvaurio suoliston seinämässä, joka altistaa myyrän toissijaisille bakteeri- ja virustartunnoille voimistaen näin Th-1-soluvastetta. Tämä voimistunut Th-1-soluvaste voisi sitten suojella metsämyyrää uusilta PUUV-tartunnoilta. Yhteistartuntoja esiintyy kaikkialla luonnossa, eikä niiden vaikutuksia pidä laiminlyödä luonnonvaraisten eläinten tarttuvia tauteja tutkittaessa. Yhteistartuntojen seuraukset voivat olla hyvinkin yllättäviä ja johtaa tahattomiin lopputulemiin esimerkiksi zoonoosien hallinnan yhteydessä. Lisää tutkimusta ja suurempia näytöskokoja tarvitaan tulosten vahvistamiseksi ja yhteistartuntojen immunologian ymmärtämisen syventämiseksi.

Avainsanat - Nyckelord - Keywords

coinfection, bank vole, Puumala hantavirus, helminth, immunology, Th-1/Th-2

Säilytyspaikka - Förvaringställe - Where deposited

HELDA – Helsingin yliopiston digitaalinen arkisto

Työn johtaja (tiedekunnan professori tai dosentti) ja ohjaaja(t) - Instruktör och ledare - Director and Supervisor(s)

Olli Vapalahti

CONTENTS

1 INTRODUCTION

Puumala hantavirus (PUUV) is a zoonotic pathogen that causes a human disease Nephropathia Epidemica (NE), known as myyräkuume in Finnish (Brummer-Korvenkontio et al. 1980). In the past five years the number of reported cases has ranged annually from 1004 to 1665 in Finland (THL 2015-2019), which is amongst the highest prevalence of PUUV in the world (Vapalahti et al. 2003). PUUV isn't transmitted from human-to-human (Brummer-Korvenkontio et al. 1980), so the risk of human infection occurs entirely from spillover infections from its natural bank vole host. In order to better understand and control human risk for PUUV, it is therefore necessary to understand the transmission dynamics of PUUV in wild vole populations. Until now research has mainly focused on a one-host-one-pathogen framework, even though in natural systems individuals are usually coinfected with multiple species of parasites (Haukisalmi et al. 1988, Al-Sabi et al. 2013, Seguel & Gottdenker 2017). Wild vole populations are heavily exposed to different helminth species (Haukisalmi et al. 1988), which are known for their ability to immunomodulate their surroundings within their hosts, altering the survival conditions of concurrent parasites (Maizels et al. 2011). Multiple parasites infesting the same host can influence the course of each other in several other ways as well.

It is known from laboratory experiments, that in vertebrates intracellular organisms such as viruses activate immune pathway T-helper 1 (Th-1) and extracellular organisms such as helminths activate immune pathway T-helper 2 (Th-2) (Mosmann & Coffman 1989, Kidd 2003). These two types of immune pathways are shown to dysregulate each other (Lucey et al. 1996). Therefore, helminth infections can alter the Th-1/Th-2 balance increasing the susceptibility to intracellular infections, or vice versa (Mosmann & Coffman 1989, Lucey et al. 1996, Infante-Duarte & Kamradt 1999, Maizels & Yazdanbakhsh 2003, Kamal & Khalifa 2006). This phenomenon is well studied in laboratory experiments, but it has been less of a focus in wildlife research despite its evident importance.

In this thesis, field surveys were conducted to investigate how Puumala hantavirus is maintained and transmitted in wild bank vole populations. Nematode burden, PUUV antibodies and PUUV RNA in lungs and kidney were measured and statistical analysis was applied to evaluate how concurrent helminth infections influence hantavirus infection

risk and viral loads in bank vole tissues. These outcomes are directly related to virus transmission in bank vole populations and the risk of human infection.

2 LITERATURE REVIEW

2.1 Wildlife zoonoses

Wildlife zoonoses are a major health concern in our globalized world. More than half of the species known to be pathogenic for humans are zoonoses (Taylor et al. 2001), meaning that they can be transmitted from vertebrate animals to people (WHO 2020a). Increasing ecotourism (Chomel et al. 2007), forest exploitation (Wolfe et al. 2005), rapid urbanization and climate change (Buliva et al. 2017) are all risk factors for the emergence and spread of infectious diseases. Meanwhile, globalization and rapid international movement of people and goods, make most infectious diseases a global public health risk (Kruse et al. 2004, Chomel et al. 2007).

The majority of zoonotic viruses originate from wildlife (Kruse et al. 2004). For example, in an extensive survey of all known zoonotic infections, only a third of zoonotic viruses were transmitted from domesticated animals making wild animals a much more likely source of animal-to-human spillover (Johnson et al. 2015). Prominent examples of such zoonoses are Nipah virus outbreaks originating from fruit bats (Chua et al. 2002), Lyme borreliosis from rodents and deer via *Ixodes* tick vectors (Steere et al. 2016), and *Salmonella* spp. from reptiles (Bertrand et al. 2008). Another great example is the novel coronavirus outbreak originating from a wet market in Wuhan, China in December 2019 causing a massive global health risk (WHO 2020b). The animal source of the coronavirus hasn't been determined yet (WHO 2020c). Human exposure to wildlife pathogens is necessary for zoonotic infections in people. Therefore, it is of utmost importance to understand the dynamics of the pathogens in their wildlife reservoir to manage and prevent the disease outbreaks in humans.

Rodents comprise almost half of global mammalian biodiversity and are distributed to nearly every corner of the planet. They often live in close contact with human populations exposing people to the pathogens they carry (Meerburg et al. 2009). For example, a study found that out of 95 well-known wildlife zoonoses, abundant wild rodents were the source of spillover infections for 58 % of the cases (Johnson et al. 2015). In addition, in the coming years rodents and other small mammals will most likely be the dominant mammals in both tropical and human-modified environments due to anthropogenic-

driven biodiversity loss (Bordes et al. 2015). In this thesis, I investigate how Puumala hantavirus, a common zoonotic infection, naturally circulates in populations of its reservoir host, the bank vole.

2.2 Bank voles

Bank voles (*Myodes glareolus*) are a widely dispersed rodent species in Europe and one of the most abundant mammals in Finland. They are distributed throughout Finland excluding northern Lapland and the archipelago (Huitu 2007).

Bank voles predominately live in dense forests, but they can also inhabit heathlands, hedges and banks if cover and food are available (Naughton 2012). They are mostly herbivorous and eat a variety of vegetarian food such as fruits, leaves, seeds, nuts and berries. Sometimes during the summer months, they also eat invertebrates such as insects and worms (Naughton 2012).

In the wild the maximum lifespan of bank voles is around 13-16 months and only some survive for over two winters (Myllymäki 1977, Innes & Millar 1994). The breeding season lasts approximately from April until early autumn and each mature female can have four to six litters annually. Gestation lasts from 16-18 days, and the litter size is typically three to five pups. Juvenile bank voles reach sexual maturity after 35 days and early-born female juveniles can reproduce already in their first summer (Naughton 2012).

In Northern Europe, bank voles, like many other small mammal species fluctuate in abundance over both seasonal and multiannual time scales. These population cycles can be divided into four phases: the increase, peak, decrease and low phase (Myllymäki 1977). Populations reach the peak phase approximately every three to five years. The winter survival of the early increase phase voles and their spring-born female offspring is high and the peak season is observed as they reproduce (Myllymäki 1977). The decrease phase starts often at the end of the peak-year breeding season and the population decline usually occurs during the winter months (Myllymäki 1977), possibly due to a combination of predation and food depletion (Huitu et al. 2003, Forbes et al. 2014). Clear consensus hasn't been reached on what regulates population cycles, despite decades of research on the topic. It is currently believed that population cycles are primarily driven

by predation, which may interact with other factors such as food depletion and infectious diseases (Klemola et al. 2000, Huitu et al. 2003, Forbes et al. 2014).

2.3 Intestinal parasites of bank voles in Finland

Like other wild animals, bank voles are heavily exposed to many different parasite groups in their natural habitat. Helminths are parasitic worms which can be grouped according to their shape into three subgroups: flukes (Trematodes), tapeworms (Cestodes) and roundworms (Nematodes) (Taylor et al. 2016). Helminths are widely dispersed in nature and most wild animals carry some types of helminths in their intestinal tract (Al-Sabi et al. 2013, Seguel & Gottdenker 2017). Tapeworms and roundworms are commonly found in wild voles and the most common helminths in bank vole are *Heligmosomum mixtum* (Nematoda) and *Catenotaenia* sp. (Cestoda) (Haukisalmi et al. 1988). These were the helminths also found in the studied bank voles. Other less common nematodes in bank voles include *Mastophorus muris*, *Capillaria* sp., *Syphacia petrusewiczi* and *Paranoplocephala kalelai* (Haukisalmi et al. 1988).

Nematodes have often a direct life cycle that involves no intermediate hosts for development from egg to infectious adult (Taylor et al. 2016). *H. mixtum* is a dioecious parasite, which has three stages of free-living larvae and fourth parasitic larval stage in its life cycle (Figure 1, Haukisalmi et al 1996). In their third and fourth larval stage they invade the gastric or small intestinal wall causing a mild inflammatory reaction, and as adult nematodes they habitat the lumen of the duodenum (Figure 1, Haukisalmi et al. 1996, Kloch et al 2015). The prevalence of *H. mixtum* in bank vole populations increases significantly with age, and the intensity of nematode burden in individual voles seems to be higher in males than in females in certain age groups (Haukisalmi et al. 1988). Female sex hormones, especially estrogen, have been previously shown to protect hosts against helminth infections whereas male sex hormones have been shown to enhance helminth development in rats and mice (Reddington et al. 1981).

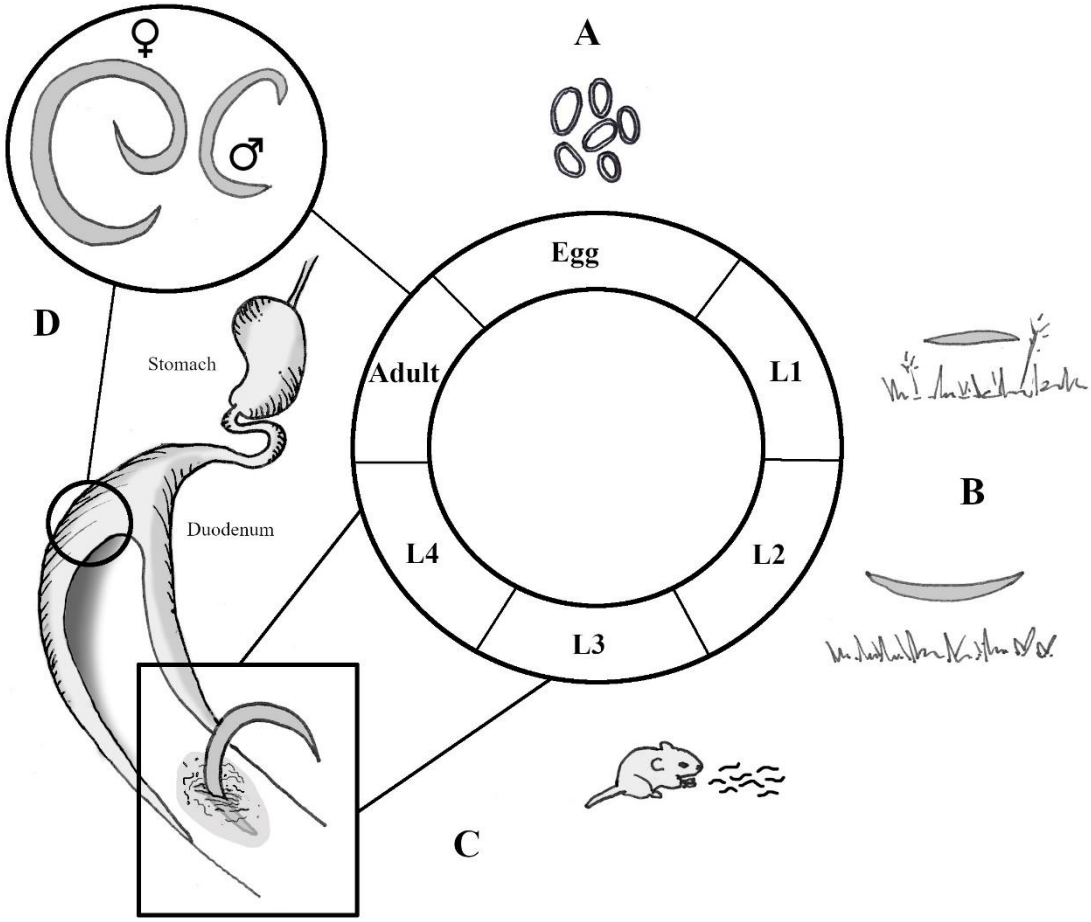


Figure 1 Life-cycle of *Heligmosomum mixtum*. (A) Eggs are shed into the environment in voles feces and when environmental conditions are optimal, larvae are hatched. (B) Larvae feed on bacteria in the soil and grows by molting and shedding the cuticle, developing from stage L1 to L3. (C) L3 is ingested by the host and the larvae buries itself in the intestinal or gastric wall where it molts twice more, developing from L3 to adult. Inflammatory reactions caused by L3 and L4 invading the intestinal or gastric wall are represented in the figure as the grey area. After L4, the nematode is subadult until it is fully grown and reaches its sexual maturity. (D) Since *H. mixtum* is a dioechious parasite, females and males can be separated from each other, male being usually smaller. In the lumen of duodenum, adult nematodes mate and females produce eggs, which are shed in feces and the process begins again.

The life cycle of cestodes is indirect, meaning that they need at least one intermediate host of another species to develop into an adult (Taylor et al. 2016). Adult tapeworms consist of a head, a neck and a chain of segments, called proglottids, that contain the genital system and eggs. The adult worms usually habitat the small intestine of the final host, and pass eggs via feces by detaching an intact proglottid from the exterior end of the chain (Taylor et a. 2016).

2.4 Hantaviruses

Helminths are not the only parasites wildlife commonly carry inside them but there are also many intracellular parasites that share the same hosts. Orthohantaviruses are enveloped RNA viruses that form their own *Hantaviridae* family within the large and diverse *Bunyavirales* order (ICTV 2018). Hantavirus virions are round, and the average diameter is approximately 80 to 120 nm (ICTV 2009). Due to their envelope, they are easily inactivated by environmental factors such as UV-radiation, heat, detergents and lipid solvents (ICTV 2009). Around the globe there are over 28 hantaviruses that cause illness in humans (Avšič-Županc et al. 2015) and additional undiscovered hantaviruses may exist since many infections go undetected especially in developing countries (Arai et al. 2007, Jonsson et al. 2010, Jiang et al. 2017). It is estimated that hantaviruses cause approximately 20 000 - 30 000 annual human infections globally (Watson et al. 2014, Jiang et al. 2017).

Hantaan virus (HTNV) was the first discovered hantavirus as the causative agent of Korean hemorrhagic fever in 1978 by Lee et al. when they managed to isolate it from its wild rodent reservoir, the striped field mouse (*Apodemus agrarius*). Other hantaviruses include Sin Nombre virus (SNV) in deer mice (*Peromyscus maniculatus*), Seoul virus (SEOV) in brown rats (*Rattus norvegicus*) and Dobrava virus (DOBV) in yellow-necked mice (*Apodemus flavicollis*) (Avšič-Županc et al. 2015). As seen here, each hantavirus has their own specific reservoir rodent species and a spillover infection to another species is usually a dead-end for the virus (Vapalahti et al. 2003). The exception is Andes virus (ANDV) in Argentina, for which person-to-person transmission was reported during an outbreak (Martinez et al. 2005).

Hantaviruses are generally divided into two groups according to their rodent reservoir, geographic distribution and the type of illness they can cause in humans: Old World hantaviruses and New World hantaviruses. Old World hantaviruses generally cause hemorrhagic fever with renal syndrome (HFRS) with mortality rates up to 12 %, and New World hantaviruses are associated with a hantavirus pulmonary syndrome (HPS) with mortality rates up to 60 % in some outbreaks (Jonsson et al. 2010). However, not all hantaviruses are pathogenic to humans and many asymptomatic or mild infections are known to occur (Ramanathan & Jonsson 2008, Jonsson et al. 2010).

2.4.1 Puumala hantavirus

Puumala hantavirus is an Old World hantavirus and the most common hantavirus infection in central and northern Europe (Latus et al. 2015). The clinical outcome for people, hemorrhagic fever with renal syndrome, was first described in the literature in Sweden already in 1934 (Kruger et al. 2001). However, researchers in Finland isolated Puumala hantavirus (PUUV) from bank voles only in the 80s (Brummer-Korvenkontio et al. 1980). The bank vole is PUUV's main reservoir species (Kanerva et al. 1998) and humans usually become infected by inhaling virus aerosols shed in bank voles' feces, urine and saliva (Forbes et al. 2018). PUUV prevalence in Finnish bank voles is high (mean annual prevalence ~ 34 %) and is affected by the annual and seasonal population fluctuations of the vole host (Voutilainen et al. 2015, Khalil et al. 2019).

In humans, PUUV causes a disease called Nephropathia Epidemica (NE), which is a mild version of hemorrhagic fever with renal syndrome (HFRS) (Kanerva et al. 1998). Symptoms typically include an acute onset of fever followed by abdominal pain, vomiting, nausea and signs of renal impairment (Kramski et a. 2009). Severe thrombocytopenia is common in severe NE cases, but bleeding complications are rare (Latus et al. 2015). Infections can also be asymptomatic, and the mortality rate is relatively low (< 1 %) (Hjertqvist et al. 2010). Annually, approximately 10 000 people in Europe (Russia included) are diagnosed with NE (Vaheri et al. 2012). Most cases occur in Russia, Finland and Sweden but they are also becoming more common in central

European countries, such as Belgium and Germany (Vapalahti et al. 2003, Avšič-Županc et al. 2015).

A correlation between the human incidence of NE and the abundance of the bank voles has been demonstrated in many parts of Europe (Heyman et al. 2007, Kallio et al. 2009, Khalil et al. 2019). As bank voles eat seeds, fruits and green plants, their food sources are directly affected by the seasonal changes in the seed production of trees such as oak, beech and acorn. Mild winters usually result in higher seed production which then leads to increased rodent population densities the subsequent year (Heyman et al. 2007, Khalil et al. 2019). It is possible to predict the upcoming NE risk up to two years ahead based on weather conditions in western and central Europe where the cyclicity occurs mainly after mast years (Heyman et al. 2007). To predict the human NE epidemics up to 18 months ahead during a given vole cycle in the northern boreal zone, a vole abundance at the beginning of that cycle may be used (Kallio et al. 2009, Khalil et al. 2019). As the density of voles increase, the virus spreads within the rodent populations and the NE cases in humans increase significantly in the following months (Kallio et al. 2009).

In bank voles PUUV infection is considered asymptomatic and persistent, like for most other hantavirus species and their respective hosts (Bernshtein et al. 1999). However, recently it has been shown that infection can impair vole winter survival, increase juvenile mortality and hempen weight gain in the host (Kallio et al. 2007, Hussein et al. 2014). It may also affect the fitness of the population by influencing the reproductive rates of the infected voles (Kallio et al. 2015).

2.4.2 Mechanisms of transmission

Infected bank voles shed PUUV in their urine, feces and saliva, and the transmission of infection between voles and from voles to humans occurs via inhaled aerosols or direct contact such as biting and grooming (Figure 2, Yanagihara et al. 1985). Virus replication and shedding peaks in voles during the first month of infection and the aerosols, in which the virus spreads, can reach a distance up to 1.5 m (Bernshtein et al. 1999). It has been shown in experimental conditions that PUUV remains infectious for 12-15 days outside of its host at room temperature (Kallio et al. 2006a), and that the materials from vole nests

can also serve as a source of infection during the first month of infection (Yanagihara et al. 1985, Bernshtein et al. 1999). In nature, environmental conditions can alter the stability of PUUV due to factors such as UV radiation and temperature changes (ICTV 2009).

As stated before, PUUVs transmission to non-reservoir species, such as human, is called a spillover infection. Several factors related to exposure risk have been identified for people, including working in forestry or on a farm, as well as chopping wood and cleaning rodent infested buildings (Makary et al. 2010). Spillover infections to people are a dead-end for PUUV as transmission from person to person is not known to occur (Vapalahti et al. 2003). Therefore, sustained transmission from the reservoir population is needed to maintain the infection and in order to control and understand the risk for human exposure, it is crucial to investigate the transmission dynamics within wild vole populations.

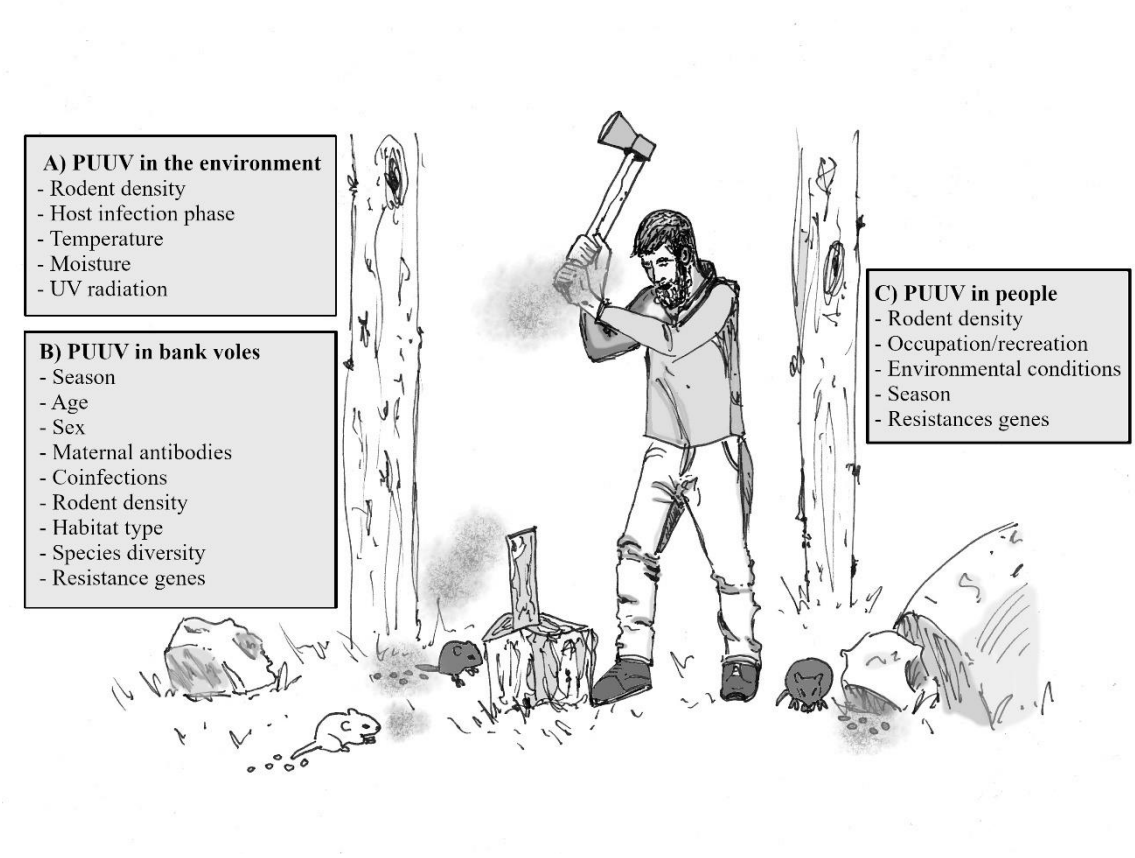


Figure 2 PUUV transmission setting. Certain factors have been shown to influence the amount and persistence of infectious PUUV in the environment (A), and the risk of infection in bank voles (B) and people (C). Dark grey voles represent the infected individuals who excrete the virus in their feces, urine and saliva. Grey clouds represent the viral aerosols that are then inhaled by people and other voles resulting in new infections (adapted from Forbes et al. 2018).

2.4.3 Sources of variation in vole infection risk

Several factors can influence the amount and diversity of parasites infecting hosts. These include individual host factors (age, sex, diet, genetic, immune response etc.), environmental factors (habitat, season), evolutionary background factors (co-evolution, parasite life cycle etc.) and the host population factors (density, predator-prey interactions etc.) (Petney & Andrews 1998). PUUV infection risk in voles and population level transmission dynamics also often vary according to these factors.

Age structure of the population affects the prevalence of PUUV since older individuals have a higher chance of being PUUV infected simply because they have existed for longer and PUUV infection is persistent (Yanagihara et al. 1985). Male voles tend to have higher prevalence of PUUV than females, which is likely to be due to greater exposure to the virus during breeding (Tersago et al. 2011). During the breeding season males cover longer distances in search of new females and have aggressive encounters with other males. It has been suggested that during the breeding season the reproductive activity of males dominate PUUV transmission dynamics while other factors have a larger role outside of the breeding season (Tersago et al. 2011). Since PUUV is transmitted mainly via direct contact and aerosols it is considered a density-dependent virus, meaning that transmission increases as vole abundance increases. It has also been shown that females can pass maternal immunity via maternal antibodies to their progeny, which gives the offspring resistance against PUUV for up to 80 days (Bernshtein et al. 1999, Kallio et al. 2006b).

2.5 Coinfections

Another source of individual variation in infection risk can be concurrent infections by multiple different pathogens and parasites. Even though in natural systems individuals are typically coinfected with several species of parasites, until now research has mainly focused on one-host-one-pathogen systems (Pedersen & Fenton 2006). To understand properly the transmission dynamics of the pathogens within the populations, further attention should be given to the mechanisms of multiparasite communities within the

hosts (Petney & Andrews 1998). The complexity of coinfections can make it problematic to predict how the infections within the host affect the course of each other. One way to understand it and to make the predictability better is to apply rules from common ecology (Pedersen & Fenton 2006, Graham 2008).

Pedersen & Fenton (2006) suggested that within-host parasite networks contain three trophic levels: host resources, the host immune system and the parasite community. Host resources include compounds parasites can feed on and living space, the host immune system works as the analog for predator, and the parasite community includes the different parasites infecting the host (Pedersen & Fenton 2006). The parasite population within-host can be controlled via resource limitation (bottom-up) or via predation when hosts' immune cells attacks and destroys pathogens (top-down) (Graham 2008). There are always three possible outcomes of a coinfection: infections can enhance each other, suppress each other or there is no affect at all (Cox 2001). An example of the bottom-up control mechanism where the other parasite suppresses the other one, could be a parasite that induces anemia and subsequently limits the growth of another parasite that requires erythrocytes (Graham 2008).

The immune systems respond to the parasite is a top-down control mechanism, and in vertebrates the antagonistic relationship between the immune pathways T-helper-1 (Th-1) and T-helper-2 (Th-2) is well studied in laboratory experiments (Mosmann & Coffman 1989, Lucey et al. 1996, Infante-Duarte & Kamradt 1999, Maizels & Yazdanbakhsh 2003, Kamal & Khalifa 2006). Th-1 directs immune responses against intracellular organisms such as viruses and Th-2 extracellular organisms such as helminths (Lucey et al. 1996, Infante-Duarte & Kamradt 1999). Th-1 cells secrete cytokines, which activate inflammatory pathways mainly via macrophage activation that is suitable for protection against intracellular parasites. Th-2 cells secrete cytokines, which upregulate antibody formation via B cells, eosinophils, mast cells and other pathways, being more suitable for protection against extracellular parasites (Romagnani 1999, Kidd 2003). Once a new pathogen has been detected, the immune pathway becomes type-1 or type-2 biased based on the type of pathogen. Type-1 pathway stimulates the maturation of Th-1 cells in a self-reinforcing "autocrine" loop, and type-2 pathway works the same for Th-2 cells (Romagnani 1999, Kidd 2003). These two pathways are shown to downregulate each other and, in some studies, helminth infections have altered the Th-1/Th-2 balance increasing the susceptibility of hosts to intracellular infections (Lucey et al. 1996, Kamal

& Khalifa 2006). Therefore, preexisting helminth infections could impair the ability of a host to resist viral infections, making them more likely to become infected and shed virus into the environment. However, this theory isn't well studied in natural settings with wildlife species.

These co-infections as a source of heterogeneity have been given little attention in recent research on the transmission dynamics of hantaviruses. One pilot study was conducted to shed light on the topic (Salvador et al. 2011). They noticed a non-significant trend that voles infected with a nematode *H. mixtum* were more likely to be PUUV positive and that the viral loads were slightly higher in these coinfected voles than in voles infected only by PUUV. These results need reinforcing since the number of PUUV positive voles was quite low (n = 21) and there was a relatively large amount of different helminth species detected compared to the overall sample pool, making it more difficult to find clear correlations between specific helminths and PUUV. In another study, helminth-PUUV coinfections have been seen to alter the immune gene expression which could cause the positive trend between PUUV and *H. mixtum* (Guivier et al. 2014). Females expressed these immune genes more strongly than males which could also partly explain why males tend to be more frequently infected with PUUV than females.

3 OBJECTIVES

The purpose of my thesis is to investigate how Puumala hantavirus is maintained and transmitted in its natural bank vole host. The main objective is to understand how coinfections shape patterns of PUUV prevalence within vole populations and thereby influence human exposure to this virus.

Based on the antagonistic relationship between the Th-1 and Th-2 immune pathways, which are upregulated by viruses and helminths, respectively, I hypothesize that: 1) PUUV infections are more likely in helminth infected voles than uninfected voles, and 2) coinfected voles' exhibit greater PUUV infection intensity.

4 MATERIAL AND METHODS

4.1 Study area and fieldwork

Cross-sectional field surveys of wild bank voles were conducted in Finland for this research. Trapping was conducted at two primary sites, around Kuhmoinen and Suonenjoki in South-Central Finland during the years 2016 - 2017 (Figure 3 and 4). Trapping was conducted during three separate trapping trips in May 2016, April 2017 and August 2017. The aim was to catch voles from multiple populations and during different seasons in order to get as much variation in the sample collection as possible. Voles were trapped in 24 different locations within these sites, which were separated from each other by distance, habitat types or a barrier such as gravel road. Voles dispersal varies between different seasons, but their territory usually covers approximately 0,5 - 1 kilometer (Henttonen, unpublished data). Although, during the breeding season especially the males can cover longer distances as part of their breeding behavior (Tersago et al. 2011). If distance between different traplines exceeded approximately 1 km, and there were no other barriers, they were considered separate locations. Coordinates of each location were marked down with a GPS device (Figure 4).

Figure 3 An Ugglan live trap set to catch bank voles in a typical forest site for this study. Red ribbons were attached to trees next to the trap to help to relocate the traps the next day.

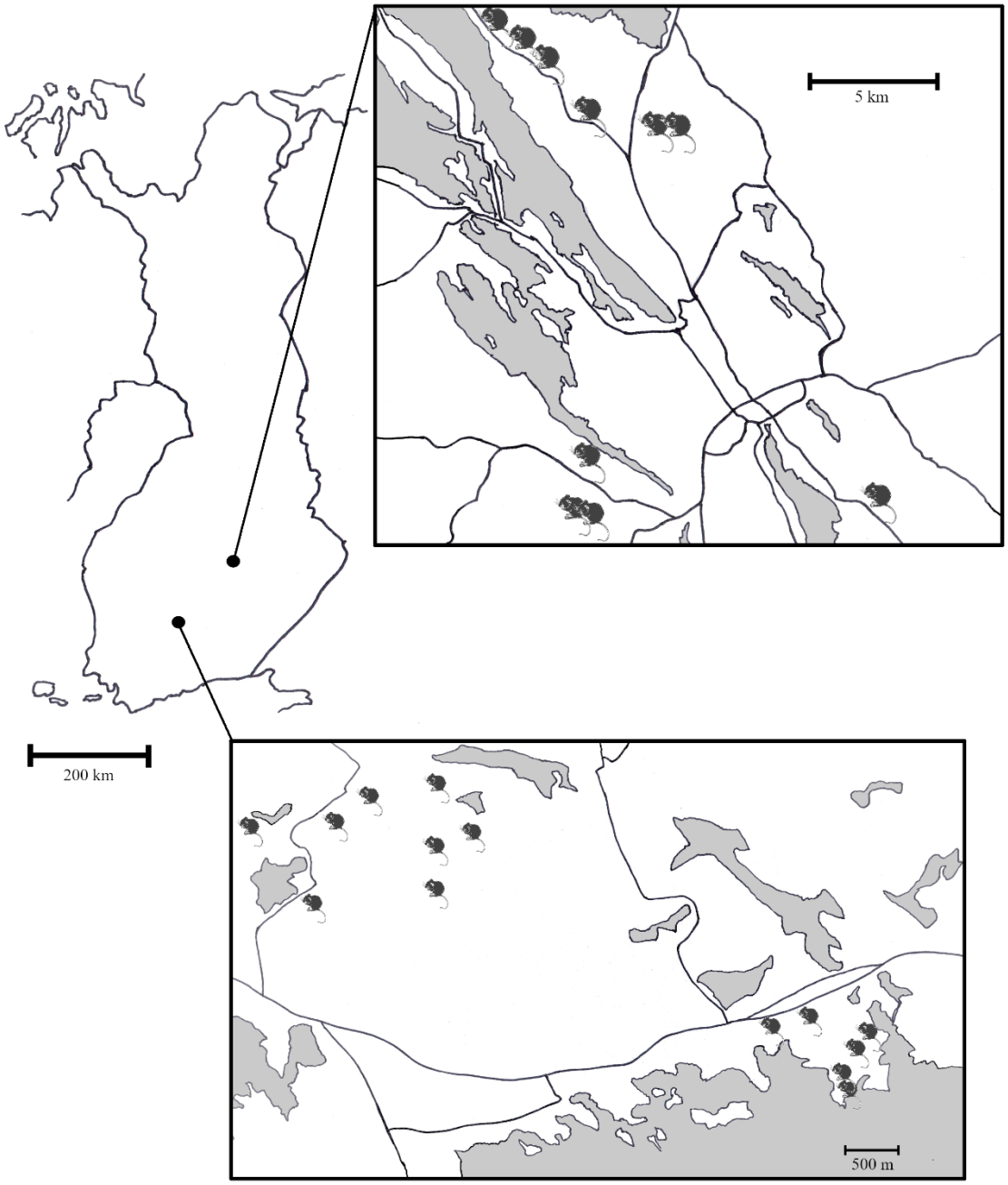


Figure 4 Trapping was conducted primarily in Suonenjoki (top) and Kuhmoinen (down). Separate traplines are marked with dark grey voles. Grey areas are lakes and paved roads are marked with black lines.

To capture voles, Ugglan live traps were baited with turnip and oat seeds and set over night. Cotton wool was also added to traps to provide warmth. Traps were set in lines, with approximately 20 traps set 10 meters apart and marked with red ribbon. Traps were set in areas where voles are usually active, such as near small nests, covered tunnels and tree roots (Figure 3). Approximately two to three trap lines were set in each location at a time and traps were checked daily in the early morning hours. Trap lines were kept in the same place for a second night if it had been successful during the first night. Baits were replaced as needed.

Trapped voles were placed in a bucket with sawdust for shelter and suitable food for nutrition and transferred to a laboratory building for processing. Blood samples were taken by using the retro-orbital blood collection route and the voles were then quickly euthanized by cervical dislocation. Each vole was assigned an identification number and its age, sex, weight, body length and reproductive status was recorded. Sexual maturity was estimated by visual observation and measurements of testes for males and uterus size for females.

Voles were dissected in a laboratory facility inside a fume hood while using adequate protective clothing including respirator mask, lab coat, plastic gloves and hair protector. Used instruments were cleaned with a scrub and then placed into a disinfectant (Virkon) for 15 minutes after each dissected vole. Organs (lymphoid tissue, lung, spleen, kidney, liver) were placed into previously marked tubes and frozen (-70°C) for later diagnostic testing. Digestive tracts were also collected and placed into marked grip-locked bags and frozen (-20°C) for later screening for helminth infections.

Digestive tracts were later unfrozen and dissected for helminths in the BSL-2-laboratory (Figure 5). For this, the digestive tract was separated into six sections: stomach, three sections of small intestine, cecum and rectum. Each section was opened with scissors and the helminths found inside were identified, counted and recorded. Helminths were identified to genus and species level based on morphology.

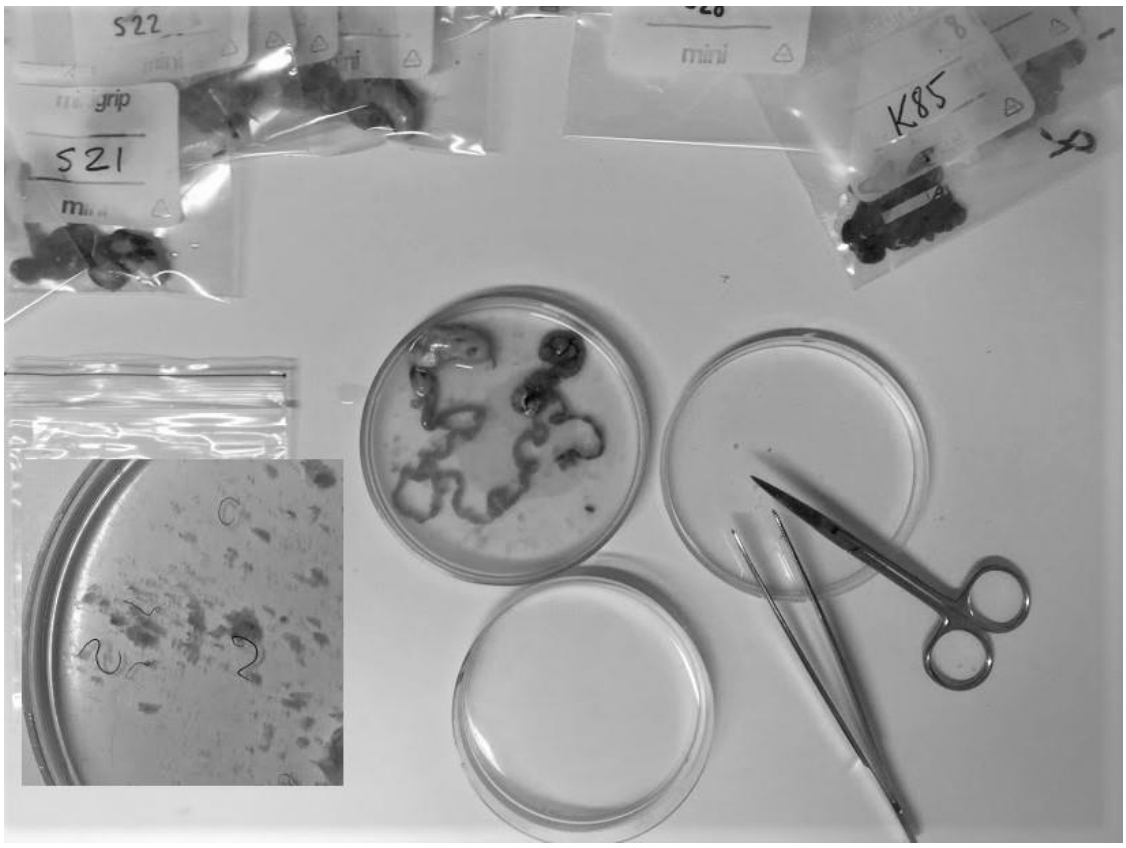


Figure 5 Dissection of the digestive tracts and count and identification of the helminths in BSL-2-laboratory. The helical shaped helminths in the left corner are the most commonly found nematodes, *Heligmosomum mixtum*, in Finnish bank voles (Haukisalmi et al. 1998). They are dark red in color and approximately 4 – 30 mm long and 1 mm thick.

All vole trapping and sampling procedures were approved by the Finnish Animal Ethics Council ESAVI/6935/04.10.07/2016. Bank voles are not protected in Finland, and animal suffering was minimized by having only experienced researchers handling live animals.

4.2 Laboratory diagnostics

4.2.1 Extraction of RNA

RNA extractions and polymerase chain reaction (PCR) assays were performed to test for the presence and quantity of PUUV RNA in vole organs. For this, the dissected tissue sample was homogenized using MagNA Lyser (four times 6000 rpm for 10 seconds) in sand and glass bead containing tubes with 800 microliters of Trisure (Bioline). The homogenized sample was then centrifuged at 20 800 rcf for 10 seconds and 600 microliters of the sample was transferred into a new marked tube. A total of 120 microliters of chloroform was added into the new tube and shaken vigorously. The samples were then centrifuged at 12 000 rcf for 15 minutes at room temperature. The colorless upper phase with the nucleic acids was transferred to a new tube with 300 microliters of 2-propanol. A volume of 0,5 ml of glycogen (50 mg/ml) was added and samples were incubated for 10 minutes at room temperature for purification. The samples were then centrifuged at 12 000 rcf for 10 minutes at room temperature. The supernatant was discarded and 800 microliters of 75 % ethanol was added, and the sample was vortexed. At this point most of the RNA precipitates were frozen (-20 °C) to continue the work later.

The RNA precipitate was then centrifuged at 8000 rcf for 5 minutes at room temperature. The supernatant was then carefully pipetted and discarded and the samples were air-dried for 10 minutes. The RNA pellet was re-suspended in 35 (kidney and lung) or 100 (spleen and liver) microliters with sterile diethylpyrocarbonate water. The solution was then incubated for 5 minutes at +60 °C before using it for PCR.

4.2.2 Quantitative reverse transcription PCR

To determine the presence of viral RNA in the vole organs, quantitative reverse transcription PCR (qRT-PCR) was used. PCR is a useful diagnostic tool in which two primers hybridize to DNA targets and the region is exponentially amplified by a DNA polymerase enzyme (Rio 2014). In RT-PCR the aim is to screen for the presence of a

specific target RNA in which primers anneal to the target RNA and an enzyme, reverse transcriptase, synthesizes complementary DNA (cDNA). cDNA is then used for the PCR reaction (Rio 2014). With real time qPCR it's possible to measure the amount of target cDNA in the sample (Ares 2014). In this study the amplification of the specific target (PUUV RNA) was measured using TaqMan measurement, in which a quenched fluorescent oligonucleotide probe is included in the PCR. When the probe binds to the specific amplification product the fluorescent dye is released from the quencher. The fluorescent is detected and correlates with the amount of DNA originally in the sample (Ares 2014).

In this study we applied a one-step qRT-PCR assay developed for the detection of PUUV S segment RNA (Niskanen et al. 2019), using AriaMx Real-Time PCR Instrumentation from Agilent with the following protocol. The reaction mix contained 2,5 µl of TaqMan™ Fast Virus 1-Step Master Mix, 0,09 µl of both forward (fw 12) and reverse (rev87) PCR primers (10 µM), 0,025 µl of PUUS-probe (10 µM), 6,295 µl of sterile water, and 1 µl of sample. The reaction was incubated at 48 ºC for 5 min for production of cDNA, followed by 3 min at 95 ºC to activate the Taq polymerase. The amplification process consisted of 45, 3 sec cycles at 95 ºC to melt double-stranded DNA and one, 30 sec cycle at 60 ºC for primer annealing and DNA extension. These reactions were carried out in on 96-well reaction plate. Ten-fold serial dilutions of PUUV S segment RNA were used to obtain the standard curve for each assay. The standard curve was used to calculate the amount of PUUV S segment RNA in each positive sample using NEBioCalculator v 1.8.0.

4.2.3 Sample standardization

To compare the viral loads between individual voles ant tissues of the same animal the amount of RNA needed to be standardized. The total RNA for each sample was measured with fluorescence spectrophotometry and the ratio between the PUUV RNA and the total RNA was counted. Spectrophotometry is a quantitative tool, which measures the light absorbed by the medium to count the number of molecules in the medium based on the Beer-Lambert law (Gore 2000). In order to specifically detect RNA molecules, RNA-binding fluorescent probes were used (Gore 2000). In this study the Quant-iT™ RNA

Assay Kit (Thermo) was used to measure the total RNA in the PUUV-positive organ samples. The assay was carried out according to the manufactures protocol.

Viral loads of organ samples were standardized by dividing the amount of PUUV S segment (ng) with the total amount of RNA (ng) in the sample. The formula is as follows:

$$PUUV_{organ}\ \% = 100 * \frac{\text{PUUV S segment ng}}{\text{Total amount of RNA ng}}$$

4.2.4 Serology

Immunofluorescence assays were performed to evaluate PUUV-specific IgG in bank vole blood based on a previously described method (Hedman et al. 1991, Kallio-Kokko et al. 2006). PUUV-infected Vero E6 cells were detached with trypsin, mixed with uninfected Vero E6 cells (1:3), washed with phosphate-buffered saline (PBS) and air-dried and fixed with acetone on microscopic slides. Vole blood samples were then diluted in PBS (1:10), added to the slides, and incubated at 37 °C for 30 min. After this, slides were washed three times with PBS and once with sterile water and incubated at 37 °C for 30 min with PBS-diluted (1:30) FITC-conjugated rabbit anti-mouse IgG (Dako Cytomation). Finally, after slides were washed three times with PBS and once with sterile water, bound IgGs were detected using a fluorescence microscope.

4.3 Statistical analyses

An abundance index for each trapping occasion was calculated based on how many traps were set for how many nights and the number of voles caught during these nights. The formula is as follows:

$$Abundace\ index = \frac{\text{Number of caught voles}}{\text{Amount of traps set} * \text{nights}}$$

Power data transformation to the fourth root was conducted for PUUV viral loads in kidney and lungs to make the data normally distributed and enable linear statistical models. Since spleen samples were only available for some of the voles, they were left out from the analyses. Statistical software Stata 15.1 was used to analyze the effects of nematode burden on PUUV seropositivity, PUUV viral loads in kidney and PUUV viral loads in lungs using multilevel mixed effects generalized linear models (MEGLM). The environmental and individual factors of sex, weight (reflecting the age of a vole) and density were included as independent explanatory variables. All two-way-interactions between these variables (sex*weight, sex*density etc.) were also included. Trapping year and location were set as random factors to account for potential inherent difference between sites. Interaction terms were removed one by one, starting from the least significant until the most parsimonious model with only significant interactions ($p > 0,05$) was reached. For dichotomous dependent variables the whole available data set was used in the analyses since the aim was to test correlations on presence or absence of PUUV RNA in voles. For continuous dependent variables instead, only PUUV positive voles were included since the purpose was to compare viral loads between infected voles.

When comparing mean nematode burdens in PUUV seropositive and PUUV seronegative voles, sample groups did not follow normal distribution but the sample size was rather large, and distribution was not extremely skewed. Therefore, more powerful Independent-Samples T test was used and carried out in IBM SPSS Statistics (Version 25) (Lumley et al. 2002). However, a Mann-Whitney U test was also carried out to validate the results.

5 RESULTS

5.1 General description of data set

A total of 129 voles were trapped across the project (Figure 6). Out of these voles, 39.5 % (51/129) were females and 60.5 % (78/129) were males. Mean body mass (reflecting age) was 21 g, and ranged from 7,9 g to 33,0 g. Body length (tip of snout to end of tail) was on average 93 mm, and ranged from 61 mm to 105 mm.

A total of 86 % (111/129) of voles were infected with the helminth *H. mixtum*, and 6,3 % (7/111) of these voles were concurrently infected with helminths belonging to the class of Cestoda. The mean *H. mixtum* infection load was 8 per infected vole and ranged from 1 to 36 per vole. Cestoda infection load averaged 2,4 helminths per infected vole and ranged from 1 to 5 per vole.

PUUV specific IgG antibodies were detected in 36 % (46/128) of voles; a blood sample of one vole was not available for the study. A total of 33 % (26/78) of the male voles were seropositive for PUUV, and 39 % (20/51) of the females were seropositive for PUUV. Two overwintered female voles were seropositive although no PUUV RNA was found in either of the two organs tested.

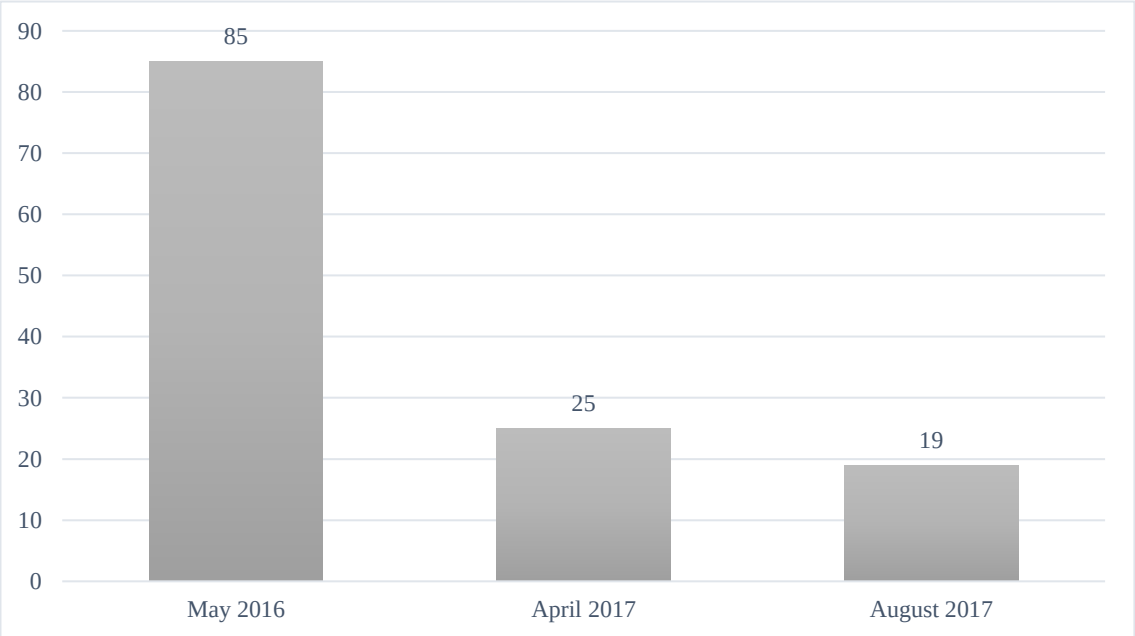


Figure 6 Number of voles trapped during different trapping trips.

Table 1 Viral loads in different organs. Proportion of PUUV RNA compared to the amount of total RNA in an organ sample.

Organ	Prevalence of PUUV	Mean viral load	Viral load range
Lung	46 % (59/129)	1,3 %	1,7 E-07 % to 41 %
Kidney	45 % (55/129)	0,012 %	2,6 E-07 % to 0,26 %
Spleen	65 %* (36/55)	0,017 %	3,1 E-08 % to 0,93 %

* *Note:* Spleens of 38 voles were not available for the study and spleens were therefore excluded from the later data analyses

Standardized PUUV S segment burdens were highest in lung samples compared to other organs (Table 1). There were 16 voles that had PUUV in their lungs but were negative for PUUV in serology, and 7 voles which had PUUV in their kidneys but not in their lungs. In total 53 % (69/129) of voles were PUUV positive at least in one of the three measurement techniques (seroconversion, kidney viral RNA, lung viral RNA).

5.2 Concurrent infections

In total 43 % (56/129) of voles were coinfected with *H. mixtum* and PUUV (positive either in kidney, lungs or serology). Out of these voles, 13 % (7/56) were coinfected with *H. mixtum,* PUUV and helminths belonging to class Cestoda. Only 4.7 % (6/129) of the voles were negative for both helminths and PUUV (negative in kidney, lungs and serology).

Table 2 Most parsimonious model to explain each response variable. Significant (p > 0,05) explanatory variables and interaction terms are bolded.

Response and source of variation	Coefficient	Standard error	*p*-value
PUUV serology			
Nematode burden	**- 0.094**	**0.042**	**0.024**
Weight	0.086	0.059	0.143
Sex	0.11	0.43	0.796
Density	2.1	2.2	0.340
Infection in kidney			
Nematode burden	0.57	0.25	0.020
Weight	0.24	0.099	0.016
Sex	- 0.24	0.43	0.578
Density	2.21	2.19	0.315
Nematode burden X weight	**- 0.027**	**0.011**	**0.016**
Infection in lungs			
Nematode burden	0.011	0.041	0.795
Weight	0.046	0.059	0.436
Sex	1.36	0.66	0.038
Density	1.80	2.21	0.417
Nematode burden X sex	**- 0.20**	**0.091**	**0.030**
Viral load in kidney			
Nematode burden	- 0.0037	0.0045	0.412
Weight	0.010	0.0077	0.180
Sex	0.0090	0.058	0.878
Density	- 0.21	0.18	0.406
Viral load in lungs			
Nematode burden	0.030	0.028	0.289
Weight	0.013	0.019	0.485
Sex	- 0.22	0.16	0.178
Density	1.85	1.04	0.076
Density X nematode burden	**- 0.37**	**0.16**	**0.017**

5.2.1 Lung analysis

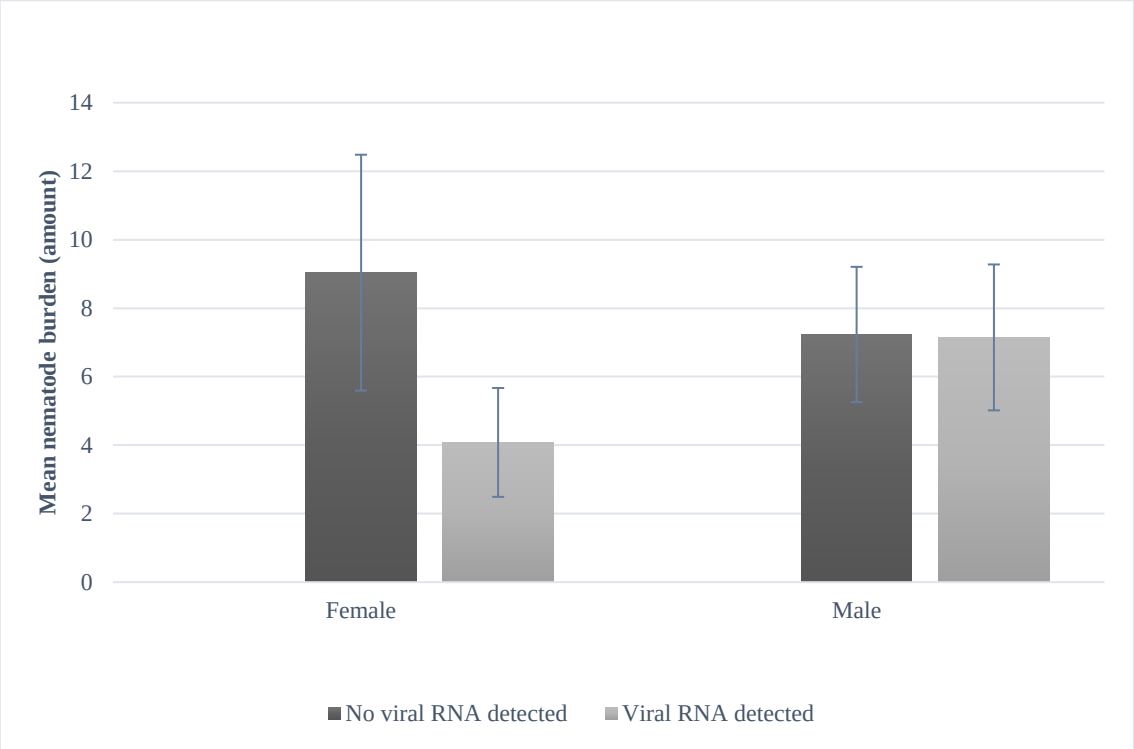


Figure 7 Mean nematode burdens in females and males in voles with PUUV RNA present or absent in the lungs. Darker grey columns represent lung-negative voles and lighter grey columns represent lung-positive voles. A significant (p = 0.030) negative correlation between the nematode burden and PUUV infection status was only observed in female voles. Error bars represent the confidence interval of 95 %.

The mean nematode burden was significantly higher in female voles which didn't have PUUV RNA present in their lungs than in female voles which had PUUV RNA present in their lungs (Table 2, Figure 7). This correlation did not occur in male voles, which had approximately the same mean nematode burden when PUUV RNA was present or absent in the lungs. Explanatory variables of nematode burden, weight, sex and density were non-significant (Table 2).

After the power data transformation, PUUV viral loads in lungs roughly followed a normal distribution (N = 59, Kolmogorov-Smirnov test Sig. = 0.061, skewness = 0.492, Std. Error = 0.311). PUUV viral loads were negatively correlated with helminth burden at high vole densities. In other words, PUUV in vole lungs was highest when the

nematode burden was low, but vole density high. Explanatory variables of nematode burden, weight, sex and density were non-significant (Table 2).

5.2.2 Kidney analysis

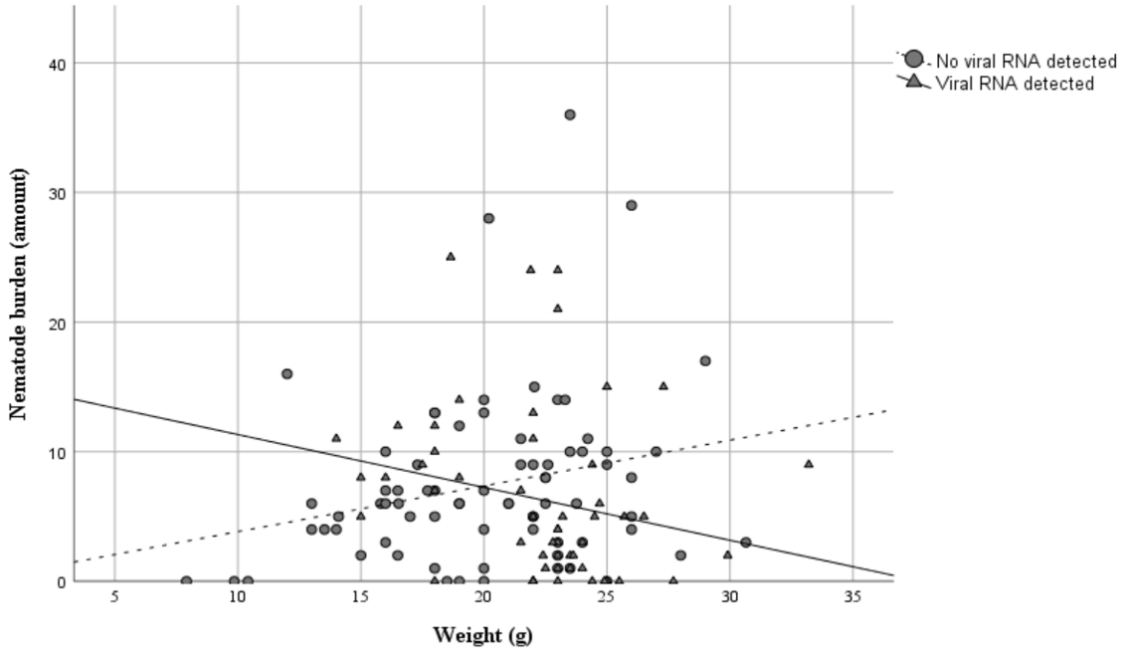


Figure 8 A significant correlation between nematode burden and weight in voles with PUUV RNA present and absent in kidneys (p = 0.016). Dashed line and circles represent kidney-negative voles whereas black line and triangles represent kidney-positive voles. Nematode burden was increasing with weight in kidney-negative voles but decreasing with weight in kidney-positive voles.

The nematode burden had a significant negative correlation on viral RNA being present in the kidney when vole mass was high (Table 2, Figure 8). Weight reflects age, so for younger uninfected voles, the nematode burden increased with age. For heavy, older PUUV infected voles, the nematode burden decreases with age. Explanatory variables of nematode burden, weight, sex and density were non-significant (Table 2).

After the power data transformation, PUUV viral loads in kidney approximately followed the normal distribution (N = 54, Kolmogorov-Smirnov Sig. = 0.028, skewness = 0.720,

Std. Error = 0.325). There were no significant findings in different sources of variation on viral loads in kidney, although nematode burden seemed to have a negative correlation on PUUV viral loads in kidney (Table 2).

5.2.3 Sources of variation in serology

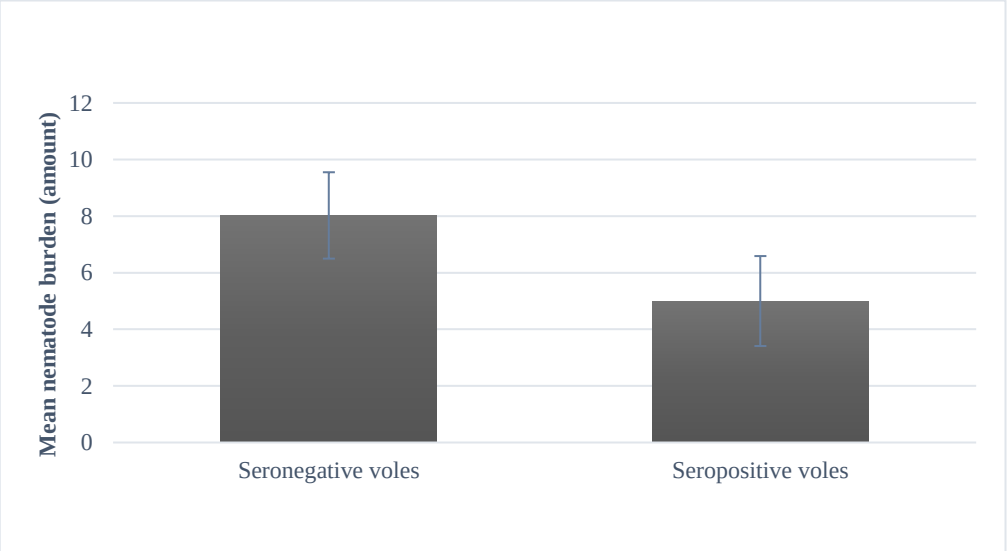


Figure 9 The mean nematode burden was higher in PUUV seronegative voles than in PUUV seropositive voles (p = 0.024). Error bars represent the confidence interval of 95 %.

The nematode burden was significantly negatively correlated with PUUV seropositivity (Table 2). In other words, when the nematode burden increased, the likelihood of voles having PUUV antibodies decreased (Figure 9). Explanatory variables of weight, sex and density were non-significant (Table 2).

The T test indicated that PUUV seronegative voles had average 3,0 intestinal nematodes more than PUUV seropositive voles (Sig. (2-tailed) = 0.012, Levene's Test Sig. = 0.274). Also Mann-Whitney U test was used to reconfirm the outcome that PUUV seronegative voles had higher mean rank (Asymp. Sig. (2-tailed) = 0.004).

6 DISCUSSION

We conducted a large cross-sectional survey of intestinal helminths and Puumala hantaviruses in wild bank voles in Finland. Our results indicate that helminths had a protective effect on voles acquiring PUUV infection and the disease burden, which was shown through serology and PCR results from kidneys and lungs. These findings are counter to our original hypotheses that helminth infections would increase the likelihood and burden of PUUV infections and have important implications for understanding the complex interactions between coinfecting parasites in natural systems.

6.1 Mechanisms of coinfection interactions

H. mixtum has a direct life cycle, which includes three stages of living larvae. The third and fourth larval stages induce an inflammation reaction and possibly some tissue damage as larvae buries itself to the host intestinal wall (Figure 1, Haukisalmi et al 1996, Kloch et al. 2015). We speculate that this could serve as a predisposing factor for secondary bacterial and viral infections, which would direct hosts' immune response towards a Th-1 pathway. This enhanced Th-1 immune response could then protect hosts from new intracellular infections such as PUUV-infection.

Some helminths have been shown to prolong their own survival by producing immunomodulatory factors that alter the intestinal environment and mediate the regulation of the intestinal inflammatory response (Behnke et al. 2001). This can also help other concurrent parasites to survive longer. *Heligmosomoides polygyrus bakeri*, a closely related nematode to *H. mixtum*, is known for its strong immunomodulatory effect in host intestines via cellular effects (Maizels et al. 2011). These cellular effects act to prevent the protective Th-2 immune pathway, which could subsequently also facilitate the upregulation of a Th-1 pathway.

6.2 Lung analysis

Lungs are the first organ to take up PUUV after inhalation by susceptible hosts, making it a good place to detect early infections that might not to be seen in the kidney (via PCR) or blood (via serology) yet. There were only two voles in which antibodies were detected in blood but no virus was detected in lungs. However, there were 16 voles which were positive for PUUV in lungs but did not have antibodies against PUUV in their blood. These voles could have been infected, as shown by PCR results, but not yet seroconverted as seroconversion can take approximately 18 days (Yanagihara et al. 1985). This can be also partly explained by late seroconversion with low doses of PUUV in bank voles, which has been seen in experimental studies for new infections (Hardestam et al. 2008). Previously, lungs have been shown to have higher concentrations of PUUV than other organs, which was also seen in this study (Korva et al. 2009). For future research, lungs could be considered the gold standard for detecting PUUV in wild voles.

Nematode burdens were significantly higher in female voles which didn't have PUUV in their lungs, but males had approximately the same average nematode burdens regardless of their PUUV infection status (Table 2, Figure 7). Previously it has been documented that the prevalence of nematodes is higher in breeding males than in females, but this difference was lacking outside the breeding season from September to April (Haukisalmi et al. 1988). Most voles for this study were caught in May (Figure 6), which is during the early breeding season. In males, maturation of subadult bank voles starts in April, a few weeks earlier than in females (Haukisalmi et al. 1988). There can be a few explanations for our finding that only females had a negative correlation between nematode burdens and the presence of PUUV in lungs. These young PUUV-negative females might start to forage outside their nests later than the more mature males of the same age, which predisposes them to higher nematode burdens (Haukisalmi et al. 1988, Boag et al. 2001, Raffel et al. 2010). Also, acquired immunity can play a part in why young tend to have higher nematode aggregation (Boag et al. 2001).

Most females were caught during summer when they are breeding. During reproduction, the maternal immune system is mildly suppressed to prevent it targeting the developing fetus, which is genetically "foreign material" (Warning et al. 2011). Reproduction is energy costly since females must reserve energy for producing milk, growing fetus and

other breeding related behavior (Randolph et al. 1977). As mounting an immune response towards parasites is also energetically costly, trade-off between resisting parasitic infection and reproduction efforts most likely exists (Gustaffsson et al. 1994, Boughton et al. 2007). This could lead to downregulation of immune responses and heavier parasite infections during reproduction for females as was seen in this study. This could also partly explain why a higher percentage of females than males, were PUUV infected.

Viral loads in lungs were highest when density was high and nematode burden low (Table 2). When vole density increases, the likelihood of voles being exposed to virus increases in a density-dependent manor. Voles with lower nematode burdens could have less damage in their intestinal wall and their immune system would subsequently be directed towards helminths via the Th-2 pathway. This could then predispose them to heavier PUUV infections compared to voles with protective nematode burdens.

6.3 Kidney analysis

PUUV detected in the kidney implies a developed infection that has spread to multiple organs after the initial infection of the lungs. PUUV is secreted in the urine, and kidneys are one of the main organs where the virus accumulates for this transmission route. Since weight roughly represents age, for young, kidney-negative voles, the nematode burden increased with age (Figure 8). This makes biological sense since the nematode burden tends to rise rapidly in juvenile voles and then starts to decrease at the age of two months (Haukisalmi et al. 1988). For old kidney-positive voles, the nematode burden was lower, which is consistent with the previous findings that nematode burden was lower in voles with PUUV present in the lungs. In general, younger voles tended to have higher nematode burdens, probably due to behavioral changes and acquired immunity (Haukisalmi et al. 1988, Boag et al. 2001, Raffel et al. 2010), and older voles tended to be more likely PUUV-kidney-positive since it’s a persistent infection and prevalence increases with age (Yanagihara et al. 1985).

Since PUUV was more likely to be present in kidney when nematode burden was low, the same mechanism could apply here as was speculated earlier. Voles with lower nematode burdens are presenting less developed immune responses towards intracellular

infections enabling PUUV to spread easier from lungs to multiple organs, including the kidneys.

6.4 Seroconversion

Since seroconversion takes some time (~ 18 days) after the initial infection (Yanagihara et al. 1985), newly infected voles might not be seroconverted at the time of antibody detection (Voutilainen et al. 2015). In general, the presence of antibodies implies that there has been an infection at some point of individuals lifespan. Since PUUV has been shown to replicate actively nearly for the lifespan of a vole (Yanagihara et al. 1985, Innes & Millar 1994), the presence of antibodies can be generalized to reflect active infection. However, in this study two overwintered female voles were seropositive but negative for PUUV in all organ screenings. These two overwintered voles might have had an older PUUV infection which is not actively replicating anymore while antibodies are persistent in the blood. Thus, serology may not be a completely reliable measure of active infection in older voles.

As would found with the analysis of viral RNA in kidneys and lungs, PUUV seronegative voles had higher nematode burdens than seropositive voles. The likelihood of a vole being seropositive for PUUV decreased as nematode burden increased.

6.5 Strengths and limitations of the study

Voles are a good model system for investigating the transmission ecology of zoonotic diseases. They are abundant mammals and reservoir hosts for many zoonotic pathogens (Meerburg et al. 2009). Due to their high abundance they can be captured relatively easy in high amounts and in reasonable time scale. This enables large sample pools with strong statistical power. In addition, voles are small and easy to handle in field work conditions. As many immunological tools are developed for laboratory model organisms such as mice, these tools and measurement techniques can often be used in other rodents in field conditions as well since they are closely related species (Bradley & Jackson 2008).

The sample size in this study was 129, which is generally enough for many parametric tests (Lumley et al. 2002) and preliminary conclusions. However, a larger sample size may have been beneficial because higher sample size decreases the possibility of few individuals with extreme characteristics to skew the results in a certain direction, although no individuals with extreme characteristics occurred in our sample pool. Also, more powerful parametric tests are possible to use when sample size is big enough even if they are not perfectly following the normal distribution (Lumley et al. 2002). In this study, the number of voles that didn't have any intestinal parasites was quite low (n = 18) and more samples should be collected in the future to reinforce these results.

Since a voles' lifespan is relatively short and their abundance fluctuates in multiannual and annual cycles, it would have been good to have samples throughout all different seasons and cycle phases. This, however, was not feasible in the study scope, and all samples were collected during breeding season which can lead to some bias in regards of differences between females and males as well as prevalence and intensity of PUUV and helminths. Nevertheless, samples were collected during multiple years and some were collected in the start of the breeding season whilst some at the end of the breeding season. This structuring of trapping helped to enhance the diversity of samples.

Intestinal helminths were identified according to their macroscopic morphology and previous knowledge of intestinal parasites of bank voles in Finland. While the helminth fauna of bank voles in Finland is well described, and largely reliable, some host-induced morphologic alterations have been reported (Waller & Thomas 1978). In this study, nematodes were identified as adult stages, which makes identification easier than earlier stages, and less invasive methods such as fecal sampling were not sensible. An alternative method more applicable to less characterized areas would have been genetic testing of different parasites, but this would also require greater expenses. There are genetic methods available to identify individual parasites but also multiple parasites concurrently (Gasser 1999, Aivelo & Medlar 2018).

7 CONCLUSIONS

This study aimed to shed light on hantavirus-helminth coinfections and highlight the complexity of coinfections in natural settings. Different parasite groups can have numerous different significant effects towards each other, and these effects should be discovered in order to fully understand the transmission dynamics of wildlife diseases. Coinfections are occurring everywhere in wildlife systems and shouldn't be neglected in the future disease management, especially when talking about zoonoses that pose a risk to humans. There can be surprising outcomes when multiple parasites share the same host, such as was found in this study, and these can lead to unintentional consequences of disease control procedures.

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